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AICAR, an activator of AMP-activated protein kinase, down-regulates the insulin receptor expression in HepG2 cells☆

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Abstract

The liver is one of the major target organs of insulin in which the expression of insulin receptor is abundant. We analyzed the effect of AICAR, an AMPK activator, on the expression of insulin receptor in a human hepatoma cell line, HepG2 cells. AICAR treatment for 48 h significantly decreased the expression of the insulin receptor protein in a dose-dependent manner, however, this same effect of AICAR was not observed in either 3T3-L1 adipocytes or CHO cells. The expression of insulin receptor mRNA also decreased after AICAR treatment. In addition, the transcriptional activity of the insulin receptor gene promoter investigated with a luciferase assay was down-regulated by AICAR treatment. Dipyridamole, an adenosine transporter inhibitor, and 5′-amino-5′-deoxyadenosine, an adenosine kinase inhibitor, blocked the effect of AICAR on the down-regulation of the insulin receptor protein, mRNA, and promoter activity. Our findings suggest, for the first time, that AMPK activation could reduce the expression of insulin receptor, at least in part, by a down-regulation of the transcriptional level, and this effect may be liver specific.

Keywords: Insulin receptor gene; AICAR; AMPK; Promoter; Transcription factor; Diabetes mellitus

Insulin initiates diverse biological effects by binding to its specific receptor on the cell surface. The binding of insulin to the α-subunit of the insulin receptor results in the stimulation of the intrinsic tyrosine kinase activity of the β-subunit, which plays an essential role in the physiological effects of insulin [1–5].

The insulin receptor gene is considered to be one of the housekeeping genes that is essential for cell growth, and it is usually synthesized at a certain level in all cells. However, the expression level of insulin receptor mRNA varies among tissues, and it is high in the liver, muscle, brain, and adipose tissue, thus suggesting the presence of tissue-specific transcriptional regulation in the insulin receptor gene.

Several nutrients and hormones have been reported to modulate the expression level of insulin receptor. A low concentration of glucose has been shown to reduce the expression level of insulin receptor mRNA [6,7]. Insulin also reduces the expression level of insulin receptor mRNA [8–10], while also altering the intracellular trafficking of insulin receptor in HepG2 cells [11]. On the other hand, glucocorticoids increase the expression of

☆ *Abbreviations:* ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMDA, 5′-amino-5′-deoxyadenosine; AMP, adenosine 5′-monophosphate; AMPK, AMP-activated protein kinase; CHO cell, Chinese hamster ovary cell; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; HNF-4α, hepatocyte nuclear factor-4α; PPAR, peroxisome proliferator-activated receptor.

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the insulin receptor mRNA and protein in several cell lines [12–15]. There are several *cis*-elements in the insulin receptor promoter, and among the several known *trans*-acting factors, Sp1 has been shown to play an important role [16]. Sp1 probably regulates the basal expression of the gene, while other factor(s) may be responsible for tissue-specific regulation. We recently reported the presence of a novel transcription factor which may be responsible for the liver-specific regulation of insulin receptor promoter [17]. However, the details regarding such tissue-specific regulation of insulin receptor have yet to be elucidated.

AMP-activated protein kinase (AMPK) is a serine–threonine kinase which is activated in response to an increase in the intracellular AMP level. AMPK was initially identified as an inactivating enzyme of HMG-CoA reductase and acetyl-CoA carboxylase (ACC) [18]. In addition, AMPK has been reported to be involved in the glucose metabolism of various tissues. Type 4 glucose transporter (GLUT4) expression and its translocation to the cell surface are accelerated by AMPK activation in cultured skeletal muscle cells [19]. In liver cells, AMPK inhibits hepatic gluconeogenesis through the suppression of phosphoenolpyruvate carboxykinase (PEPCK) gene expression [19,20].

5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) is one of the activator reagents of AMPK. AICAR is phosphorylated in cytosol by adenosine kinase and then is converted to AICAribotide (ZMP), which mimics AMP and activates AMPK [21]. AMPK regulates the expression of various genes that are involved in the glucose and lipid metabolism through an insulin-independent mechanism, and it thus ameliorates the glucose metabolism. In this study, we investigated the effect of AICAR on the expression of insulin receptor, which is the key molecule of the insulin signaling pathway.

Materials and methods

Reagents and chemicals. AICAR was purchased from Toronto Research Chemicals (Canada). Dipyridamole (adenosine transporter inhibitor) and 5′-amino-5′-deoxyadenosine (adenosine kinase inhibitor) were purchased from Sigma Chemical (St. Louis, MO). Anti-β-subunit antibody of insulin receptor (anti-IR-β) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt antibody, anti-phospho AMPK (Thr 172) antibody, and anti-phospho acetyl-CoA carboxylase (Ser 79) antibody were purchased from Cell Signaling Technology (Beverly, MA). Anti-AMPK α antibody was purchased from Upstate Biotechnology (Lake Placid, NY). All reagents and chemicals were of analytical grade.

Cell culture. HepG2 cells and Huh7 cells, both of which are derived from human hepatoma, or Chinese hamster ovary (CHO) cells were maintained in either a DMEM (Sigma) or an F-12 medium (Invitrogen, Carlsbad, CA), respectively, in a 5% CO_2 incubator at 37 °C.

Western blot analysis. Whole cell extracts were prepared as described previously [22]. Briefly, the cell pellet was resolved in lysis buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2 mM Na_3VO_4, 20 mM $Na_4P_2O_2$, 100 mM NaF, 1% NP-40, 2 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg aprotinin/ml, and 10 μg leupeptin/ml] and sonicated with the Bioruptor device (Cosmo Bio, Japan). Insoluble protein was removed by centrifugation at 15000 rpm for 20 min in a microcentrifuge. The extract was then resolved directly in SDS–polyacrylamide gel after boiling in Laemmli–SDS sample buffer. Fifty microgram protein samples were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and then were transferred to the PVDF membrane. The membrane was subjected to Western blotting with a BM Chemiluminescence Blotting Substrate (Roche Molecular Biochemicals, Indianapolis, IN) according to the instructions provided by the manufacturer. The immunoreactive bands were visualized by an enhanced chemiluminescence and then were quantified by a densitometric analysis (NIH image soft).

Real-time RT-PCR. Total RNA was extracted from HepG2 cells treated with or without AICAR (0.1–1.0 mM) and then was used for reverse transcription to amplify cDNAs using Revertra Ace (TOYOBO, Japan). Real-time PCR with SYBR green was carried out using a LightCycler instrument (Roche). The following primer sets were used to amplify the insulin receptor cDNA, forward primer 5′-CTCGCCC ATGATTTTACTG-3′, reverse primer 5′-CAGAAGAAGTGGTGA AGAC-3′, respectively.

Construction of the insulin receptor promoter luciferase gene fusion plasmid. 1.5 kb fragment of the human insulin receptor gene promoter, originating from the *pSphI-00CAT* vector [16], was inserted into the *Hin*dIII site of pGL3-basic vector (Promega, Madison, WI), and this was designated as pGL3-1.5kIRP.

Transfection. HepG2 cells (5×10^5 cells) were plated in 6-well culture plate dishes and then were incubated in the serum-free media for 12 h before transfection. One microgram of plasmid was transfected with FuGENE6 Transfection Reagent (Roche). After 5 h of transfection, the culture media were removed and then media supplemented with or without AICAR were added to each well. The stimulation media were changed every 24 h.

Luciferase assay. The luciferase activity of the constructs described above was assayed in HepG2 cells. 0.9 μg of luciferase plasmid was co-transfected with 0.1 μg pRL-SV40 (Promega) as an internal control. Cell lysates were prepared after 48 h of transfection according to the manufacturer's instructions (Promega) and then were subjected to a luciferase assay using the Dual Luciferase Assay Kit (Promega). The luciferase activity was represented by relative light units (Firefly/*Renilla*). All assays were performed in duplicate.

Statistical analysis. The data are expressed as means ± standard error of the mean (SEM). Any differences between two groups were evaluated by unpaired Student's *t* test. A value of $p < 0.05$ denoted the presence of statistical significance.

Results

AICAR reduced the expression of insulin receptor protein

HepG2 cells were treated with various concentrations of AICAR (0.1–1.0 mM) for 12, 24, and 48 h, respectively. After each time point, the cells were harvested and the total cell lysates were extracted. A Western blot analysis was performed to investigate the expression level of insulin receptor protein. Although the expression level of the insulin receptor β-subunit (IR-β) did not change at 12 h, it decreased after AICAR treatment at 48 h (Fig. 1A). The expression level of IR-β significantly decreased with 0.25, 0.5, and 1.0 mM of AICAR at 48 h to 50%, 53%, and 46% of the control, respectively. At 24 h, the expression of IR-β also tended to decrease with 1.0 mM AICAR to 55% of control, although no statistical significance was observed ($p = 0.06$). The protein